Antitumor and anti-angiogenic activity of *Ganoderma lucidum* polysaccharides peptide

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KEY WORDS *Ganoderma lucidum*; polysaccharides; sarcoma 180; adenocarcinoma; lung neoplasms; lymphocyte proliferation; peritoneal macrophages; angiogenesis; nude mice

ABSTRACT

AIM: To investigate the antitumor and anti-angiogenic activity of *Ganoderma lucidum* polysaccharides peptide (GLPP). METHODS: Antitumor effect of GLPP was observed in tumor-bearing mice *in vivo*. At the same time, the effects of GLPP on proliferation of tumor cells and human umbilical cord vascular endothelial cell (HUVEC) were detected by MTT assay *in vitro*. Subsequently, spleen lymphocytes proliferation of nude mice was stimulated by LPS or ConA. To investigate the anti-angiogenic effect of GLPP, GLPP 80µg per disc and GLPP-treated serum 10µL per disc were added to the chick chorioallantoic membrane (CAM) respectively *in vivo*. RESULTS: GLPP 50, 100, and 200 mg/kg inhibited growth of Sarcoma 180 in BALB/c mice markedly by 35.2%, 45.2%, and 61.9%, respectively. GLPP which was directly added to the cultured medium did not inhibit PG cell proliferation *in vitro*; but GLPP-treated serum 50, 100, 200 mg/kg potently inhibited PG cell proliferation by 22.5%, 26.8%, and 30.3%, respectively; and reduced the xenograft (human lung carcinoma cell PG) in BALB/c nude mice greatly *in vivo* by 55.5%, 46.0%, and 46.8%, respectively. Lymphocytes proliferation of nude mice could be stimulated by LPS 5mg/L but not by ConA 2.5mg/L, indicating that GLPP could not promote the T lymphocyte proliferation and neutral red phagocytosis of peritoneal macrophages of nude mice. The CAM assay showed that GLPP and GLPP-treated serum had anti-angiogenic effect. GLPP (1, 10, and 100 mg/L) inhibited HUVEC proliferation *in vitro* with the inhibitory rate of 9.4%, 15.6%, and 40.4%, respectively. CONCLUSION: GLPP has antitumor and anti-angiogenic activity. The anti-angiogenesis of GLPP may be a new mechanism underlying its anti-tumor effects.

INTRODUCTION

*Ganoderma lucidum* (Leyss, ex Fr) Karst (*Gl*) has been widely used as a medicine to promote health and longevity in China for thousands of years. The *Gl* and its components were reported to be effective in the treatment of chronic hepatopathy, hypertension, hyperglycemia and neoplasia under modern pharmacological research in recent 30 years. However, the most attractive nature of the *Gl* is its anti-tumor effect, which was demonstrated to be associated with its polysaccharides fraction. A series of experiments have demonstrated that *Ganoderma lucidum* polysaccharides peptide (GLPP) could stimulate host immune functions. Macrophages and lymphocytes could secret anti-tumor cytokines such as TNF-α and interferon-γ (IFN-γ) to inhibit the tumor cells proliferation. So, the antitumor effect of GLPP was supposed to be the result of en-
hanced immune function.

Several lines of direct evidence showed that angiogenesis was essential for the growth and persistence of solid tumors and their metastases[9-11]. In this study, we investigated the influence of GLPP on tumor and angiogenesis, and found anti-angiogenesis may be a new mechanism of anti-tumor effect of GLPP.

MATERIALS AND METHODS

Animals  Inbred male 6-8-week-old (body weight 18-22 g) BALB/c (H-2 d) mice (Grade II, Certificate No scxk11-00-0004) and BALB/c nude mice (Grade III, Certificate No scxk11-00-0010) were purchased from the Department of Experimental Animals, Health Science Center, Peking University, Beijing, China. All procedures were in accordance to the Institute Ethical Committee for Experimental Use of Animals.

Drugs  GLPP were isolated from boiling water extract of the fruiting body of *Ganoderma lucidum* (Leyss ex Fr) Karst (Gl), followed by ethanol precipitation, dialysis and protein depletion using Sevag method. It was a kind of polysaccharides peptide with Mr 512 500, and the ratio of polysaccharides of peptides is 94.8 %:5.2 %. The polysaccharides consisted of D-rhamnose, D-xylose, D-fructose, D-galactose, and D-glucose with molar ratio of 0.549:3.614:3.614:3.167:0.556:6.89 and linked together by β-glycosidic linkages. It is a hazel-colored and water soluble powder, kindly provided by Fuzhou Institute of Green Valley Bio-Pharm Technology.

Antitumor experiment in tumor-bearing mice  Murine Sarcoma 180 (S180) was purchased from Beijing Tumor Institute and maintained by weekly transplantation of the tumor cells into the peritoneal cavity of mouse. Human lung carcinoma cell line (PG) was purchased from Department of Pathology, Peking University Health Center. PG cells were cultured with 10 % FCS RPMI-1640 in a humidified environment at 37 ºC, 5 % CO2. Tumor cells (Sacoma 180 with 2×106/mouse or PG cells with 2×106/nude mouse) were injected into the axillary fossa of the mice right foreleg. The mice were divided into 4 groups randomly. Different doses of GLPP 50, 100, and 200 mg/kg were given intragastrically once a day. The BALB/c mice implanted sarcoma 180 were killed 10 d later and the BALB/c nude mice implanted PG cell were killed 33 d later and the tumors were cut and weighted. At the same time, blood samples were collected from the orbital vein and the serum (GLPP-treated serum) was separated and filtered, then stocked at -70 ºC.

PG cells proliferation assay  PG cells were washed with PBS and were dispersed in 0.125 % trypsin-0.02 % edetic acid solution. A cell suspension (2×105 cells/L) was made with 10 % FCS RPMI-1640, planted onto 96-well culture plates (0.1 mL/well), and incubated at 37 ºC, 5 % CO2 for 24 h. The media was replaced with 0.1 mL of RPMI-1640 +5 % FCS and the GLPP 0, 0.1, 1, 10, 100 mg/L or 1:2 diluted GLPP-treated serum 0, 50, 100, 200 mg/kg was applied, respectively. After 48 h, cells proliferation was estimated based on the cellular reduction of tetrazolium salt MTT[12] by a Microplate reader (BIO-RAD, Model 550), using a test wavelength of 540 nm.

Preparation of spleen lymphocytes of BALB/c nude mice  Mice were killed and the spleens were chopped with two slides and filtered over a fine nylon mesh. Cells were washed three times in Hanks’ balanced salt solution. Cells were finally suspended in 10 % FCS RPMI-1640 supplemented with benzylpenicillin 100 kU/L, streptomycin 100 mg/L. Microplate wells received spleen cell suspension (2×107/well) added with ConA 2.5 mg/L or LPS 5 mg/L, and incubated at 37 ºC, 5 % CO2 for 72 h. Cell proliferation was estimated based on the method of MTT.

Neutral red phagocytosis assay of peritoneal macrophages  Macrophages were obtained from the peritoneal exudates[13]. Mice peritoneal exudates cells (PECs) were harvested by peritoneal lavage using cold Hanks’ solution. PECs were washed twice and resuspended in a RPMI-1640 medium containing 10 % FCS. Peritoneal macrophages were further isolated from the PECs by incubating the PECs (2×107/well) in a 96-well plate at 37 ºC, 5 % CO2 for 2 h in a humidified atmosphere to allow peritoneal macrophages for adherence. The supernatants were discarded and 100 µL 0.075 % neutral red were added and incubated for another 1 h. Cells were then washed with PBS for three times and incubated with cell lysis buffer (1 mol/L acetic acid: ethanol=1:1) overnight. The result was recorded with a spectro-photometer, using a test wavelength of 540 nm.

Chick chorioallantoic membrane (CAM) assay[14-16]  Three-day-old fertilized white Leghorn eggs were cracked, and embryos with intact yolks were placed in petri dishes. After 3 d of incubation (37 ºC and 3 % CO2), a Glass Microfibre filters disc (Whatman) containing GLPP-treated serum or GLPP was applied to
the CAM of individual embryos. After 48 h of incubation, embryos and CAMs were observed with a stereomicroscope. The positive anti-angiogenic effect was scored when microvessels around the disc were obviously reduced after sample application or an obvious blockage of CAM vessel development was observed.

Human umbilical cord vascular endothelial cell (HUVEC) culture and proliferation assay HUVEC was isolated as the method of Jaffe et al.[17]. The HUVEC detached by 0.1% type I collagenase (Gibico) were resuspended in complete DMEM which contained 20% new born calf serum (BCS), endothelial cell growth supplement (ECGS) 50 mg/L, insulin 0.4 kU/L, streptomycin sulfate 100 mg/L, benzylpenicillin 100 kU/L. The cell suspension was then seeded on gelatin-coated 6-well culture plates (Costa, America) at a density of 4×10^7 cells/L and incubated under 5% CO_2 at 37 ºC. Cultures were fed with fresh medium every 48 h. The typical cobblestone morphology was observed using an inverted microscope (OLYMPUS). For the proliferation assay, HUVEC were dispersed in 0.05% trypsin-0.02% edetic acid solution. Cell suspension (4×10^7 cells/L) was made with DMEM+20% BCS, planted onto gelatinized 96-well culture plates (0.1 mL/well), and incubated at 37 ºC, 5% CO_2 for 24 h. The media was replaced with 0.1 mL of DMEM+5% BCS and GLPP 1, 10, and 100 mg/L was added, respectively, and incubated at 37 ºC, 5% CO_2 for 48 h. Cell proliferation was estimated based on the method of MTT.

Statistical analysis Statistical significance was determined by one-way ANOVA employing the computer SPSS statistic package. P<0.05 was considered significant.

RESULTS

Antitumor effect of GLPP on locally implanted sarcoma 180 in BALB/c mice in vivo GLPP 50, 100, 200 mg/kg inhibited growth of Sarcoma 180 by 35.2%, 45.2%, and 61.9%, respectively (Tab 1).

Effect of GLPP on proliferation of PG cells in vitro GLPP did not inhibit PG cell proliferation in vitro when was added directly to the cultured medium; but GLPP (50, 100, and 200 mg/kg)-treated serum markedly inhibited PG cell proliferation by 22.5%, 26.8%, and 30.3%, respectively (Tab 2).

Antitumor effect of GLPP on locally implanted PG human lung carcinoma cell in BALB/c nude mice

Statistical analysis

in vivo GLPP 50, 100, and 200 mg/kg inhibited the xenograft (human lung carcinoma cell PG) in BALB/c nude mice markedly in vivo with inhibitory rate of 55.5%, 46.0%, and 46.8%, respectively (Fig 1, Tab 3).

Lymphocyte proliferation assay Lymphocytes of nude mice treated with or without GLPP could be stimulated by LPS 5 mg/L but not by ConA 2.5 mg/L (Fig 2).

Neutral red phagocytosis assay of peritoneal macrophages GLPP 50, 100, and 200 mg/kg on nude mice did not have influence on neutral red phagocytosis (Tab 4).

GLPP inhibits angiogenesis The microvessels around the disc were obviously reduced at 48 h after 80 µg GLPP per disc or 10 µL GLPP (50 mg/kg)-treated serum/disc application indicating there was potent inhi-
bition of angiogenesis (Fig 3). There was no evidence of toxicity in any of the chick embryos tested.

**HUVEC proliferation assay** GLPP 1, 10, and 100 mg/L directly inhibited HUVEC cell proliferation in vitro with inhibitory rate of 9.4 %, 15.6 %, and 40.4 %, respectively (Fig 4).

**DISCUSSION**

In the present study, we investigated the antitumor activity of GLPP, a purified polysaccharide isolated from *Ganoderma lucidum*. The results showed that GLPP inhibited the growth of implanted Sarcoma 180 markedly, as the same as prior[5]. On the basis of the preceding in vivo results, we added GLPP or GLPP-treated serum directly to the PG Human lung Carcinoma cells. Unexpected results showed that GLPP could not inhibit their proliferation when it was added directly to the tumor cell cultures. While, GLPP-treated serum 50, 100, 200 mg/kg could markedly inhibit PG cells proliferation indicating that GLPP had no direct cell toxicity in tumor cells. And some experiments had proved that GLPP promoted TNF-α and IFN-γ release from immunocytes[4,5,18]. TNF-α and IFN-γ play important roles in suppressing tumor cell growth and inducing

**Tab 3. Antitumor effect of GLPP on PG carcinoma xenograft in BALB/c nude mice in vivo.** *n*=5. Mean±SD. *P<0.01 vs NS.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose/ mg·kg⁻¹</th>
<th>Body weight/g</th>
<th>Tumor weight/g</th>
<th>Tumor volume index/%</th>
<th>Inhibitory rate/%</th>
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<tr>
<td>NS</td>
<td>-</td>
<td>24.4±1.7</td>
<td>2.6±0.8</td>
<td>10.9±3.5</td>
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<tr>
<td>GLPP</td>
<td>50</td>
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<td>1.2±0.6</td>
<td>4.9±2.5</td>
<td>55.47</td>
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<tr>
<td></td>
<td>100</td>
<td>22.7±1.5</td>
<td>1.4±0.7</td>
<td>6.3±3.2</td>
<td>46.04</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>22.1±2.5</td>
<td>1.4±0.6</td>
<td>6.4±2.9</td>
<td>46.79</td>
</tr>
</tbody>
</table>

**Fig 2. Lymphocyte proliferation assay of the nude mice.** *(×400).* *n*=6. Mean±SD. *P<0.01 vs RPMI-1640.

**Tab 4. The influence of GLPP on phagocytosis of peritoneal macrophages of the nude mice.** *n*=10. Mean±SD.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose/mg·kg⁻¹</th>
<th>OD 540 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-1640</td>
<td>-</td>
<td>0.84±0.17</td>
</tr>
<tr>
<td>GLPP</td>
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<td>0.86±0.08</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.86±0.10</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0.84±0.10</td>
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apoptosis of tumor cells. Many scholars had suggested that the anti-tumor effect of GLPP was mainly mediated by the host immunological mechanism. So we wanted to observe whether or not GLPP still has anti-tumor effect on immune-default nude mice.

We observed that at GLPP 50, 100, 200 mg/kg also inhibited the xenograft (human lung carcinoma cell PG) in BALB/c nude mice markedly in vivo. The lymphocytes of nude mice could be stimulated by LPS but not by Con A suggesting GLPP could not promote the T lymphocyte proliferation of nude mice. GLPP had no effects on neutral red phagocytosis of peritoneal macrophages in nude mice.

The above results demonstrated that the cell-mediated immunity was default in nude mice, but GLPP still has high anti-tumor activity on the mice, which indicated that other anti-tumor pathway was involved in anti-tumor effects of GLPP.

Tumor growth and metastasis is angiogenesis-dependent. To stimulate angiogenesis, tumors upregulate the production of a variety of angiogenic factors, including fibroblast growth factors (aFGF and bFGF) and vascular endothelial cell growth factor/vascular permeability factor (VEGF/VPF)\(^{[19]}\). Many malignant tumors, however, also generate inhibitors of angiogenesis\(^{[20]}\), including angiostatin\(^{[21]}\) and thrombospondin\(^{[22]}\). It is becoming clear that the angiogenic phenotype is the result of a net balance between these positive and negative regulators of neovascularization\(^{[21-23]}\). Anti-angiogenesis might represent an important mechanism underlying anti-tumor activity.

To test the ability of GLPP to inhibit angiogenesis in vivo, we used the chick chorioallantoic membrane (CAM) assay. GLPP or GLPP-treated serum potently inhibit angiogenesis. To determine whether or not GLPP had effect on the endothelial cell proliferation, HUVEC proliferation was measured. The results showed that GLPP directly inhibited HUVEC cell proliferation in vitro in accordance with our primary results that GLPP induced endothelial cell apoptosis.

There are two classes of angiogenesis inhibitors – ‘direct’ and ‘indirect’. Direct angiogenesis inhibitors prevent vascular endothelial cells from proliferating, migrating or avoiding cell in response to a spectrum of pro-angiogenic proteins\(^{[24]}\). Indirect angiogenesis inhibitors generally prevent the expression of or block the activity of a tumor protein that activates angiogenesis, or block the expression of its receptor on endothelial cells. Many of these tumor-cell proteins are the products of oncogenes that drive that angiogenic switch\(^{[25,26]}\).

Cytokines IFN-\(\gamma\), IL-12, and IP-10 (interferon-inducible 10) could inhibit angiogenesis in vivo or in vitro\(^{[27,29]}\). IL-12 and IFN-\(\gamma\) also regulated the expression of tumor VEGF\(^{[30]}\). GLPP improved host immune functions and augmented cytokines production such as interferon-\(\gamma\) (IFN-\(\gamma\)) and IL-12\(^{[31]}\). So the angiogenic activity of GLPP may be dependent on both pathways: direct and indirect.

In conclusion, anti-angiogenesis of GLPP may be one of the mechanisms in its anti-tumor effects.
REFERENCES


